

Anticancer Potential of Biosynthesized Chitosan Nanoparticles from *Bacillus Subtilis* Against A549 Lung Cancer Cells

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ABSTRACT

Background: Chitosan nanoparticles (CNPs) have gained significant attention in biomedical research due to their biocompatibility, biodegradability, and potential anticancer properties. The biosynthesis of CNPs using bacterial sources such as *Bacillus subtilis* offers an eco-friendly and efficient approach for nanoparticle production, which may enhance their therapeutic applications in oncology. **Objective:** This study aimed to evaluate the ability of *Bacillus subtilis* to biosynthesize carbon nanotubes (CNPs) and investigate their anticancer effects against A549 lung cancer cells. **Methods:** Twenty *Bacillus subtilis* isolates were obtained from various sources and screened based on morphological characteristics and VITEK system identification for their ability to synthesize CNPs. The most efficient isolate was selected for nanoparticle biosynthesis. CNPs were prepared by adding 1 mg/mL of chitosan to the bacterial culture medium, followed by centrifugation at 4500 rpm for 15 minutes to separate the synthesized nanoparticles. The nanoparticles were characterized using X-ray diffraction (XRD), which showed diffraction peaks between 19.5° and 24°, and atomic force microscopy (AFM), which revealed an average particle size of 47.15 nm. Fourier-transform infrared spectroscopy (FTIR) confirmed the presence of functional groups and molecular bonding patterns in the biosynthesized CNPs. **Results:** The anticancer potential of CNPs was assessed against A549 lung cancer cells using various concentrations (1000, 500, 250, 125, 62.5, and 31.25 µg/mL) over 72 hours. The cytotoxic effects were observed with a dose-dependent reduction in cell viability, with cell death percentages recorded at 58.7%, 34.3%, 30.3%, 22.3%, 15.5%, and 9.9%, respectively. The IC50 value was determined to be 2011 µg/mL. Morphological analysis of A549 cells post-treatment indicated significant structural changes, including membrane damage, cell detachment, and disruption of monolayer integrity. **Conclusion:** The biosynthesized CNPs from *Bacillus subtilis* exhibited notable cytotoxic effects against A549 lung cancer cells, highlighting their potential as a natural nanotherapeutic agent. Further research is recommended to investigate their molecular mechanisms, optimise dosage, and evaluate their in vivo efficacy for potential clinical applications in cancer treatment.

Keywords: *Bacillus Subtilis*, Chitosan, Biosynthesized Chitosan Nanoparticles, A549 Cell Line

INTRODUCTION

Lung cancer is considered the second most common cancer in the world. It leads to death in most cases. By 2020, the number of cases reached 1.8 million, with deaths derived from lung cancer accounting for 18% of all cases. Five-year survival rates reached seven to twenty-five per cent of lung cancer cases. It became less than other essential cancers, and due to the increased rate of the spread mortality for lung cancer, it is considered very similar to that of its occurrence, which stimulates the burden of disease damage globally [1-3]. The studies previously registered the directions of lung cancer in a territory or country through offered introduced the world trends from registries data of lung old cancers [4, 5] thus, research on distribution related to danger factors for lung cancer as well as the newest fleeting in lung cancer styles, the occurrence of lung cancer, mortality cases which assist to describe distribution geographically, detect high dangers in groups of the population diagnosis of the high-risk population and notify the progress of its intervention and prevention. On the other hand, the results might be related to the future possibility of prevention in addition to early identification of lung cancer [6]. The cases of lung cancer are more common than other cases of cancer based on diagnosis around the world, reaching approximately 12.4 % of all diagnosed cases of cancer globally, causing death related to cases of cancer. So, American Cancer Society (ACS) referred to estimate new cases of lung cancer that occur annually to be higher than 234,000 and especially in US more than 154,000 cases of lung cancer, rely on the statistical reports of global cancer in the year 2020, the lung cancer

is remaining one of the causing for death in the world, about approximately 1.8 million cases of death [1]. *B. subtilis* considered one, of the primary sources and is classified as Generalized Recognized as Safe (GRAS) according to Food and Drug Administration (FDA) for probiotics, a species that represents a biosynthetic remarkable potentially [7] also secretions different secondary metabolites as lipopeptides and enzymes which give wide spectrum activities of anticancer, antimicrobial, and antineoplastic [8-10]. The backbone of CS that contains active groups that make it suitable to adaptive via ways of chemical and physical to appropriate construct used in the purposes of biological importance as adhesion on mucous membranes, fail of efflux pump, targeted for colon, to improve of permeation as well as some features belong to the functions of amino group in the structure of chitosan [11].

MATERIALS AND METHODS

Selection of Active Isolate for Synthesis of CNPs

Twenty isolates were diagnosed relying on the morphology appearance of the colony in the medium appeared as creamy white cottony or waxy as well as microscopical examination that observed purple color, rod form or bacilli as gram-positive bacteria and confirmed detection by VITEK 2 Compact System apparatus, also it was used a symbol S₂ for an effective isolate with and it was her utilized to production for CS.

Large Amount Prepared of CNPs by Using of *B. subtilis*

An active isolate that was selected and cultured in a broth of brain heart infusion and placed in a shaking incubator for 72 hours at 37°C under suitable and controlled sterilized conditions, where it was prepared and taken of supernatant and it was increased period of incubation to allow the formation of biomass, then collected of supernatant in the bottles and placed in centrifugation at fifteen minutes on 4200 rpm to completion of the separation and obtained a supernatant without any cells by collected in pellets to removed then the supernatant was used for the synthesis of CNPs after addition CS and acetic acid and placed in a flask that it was inoculated with selection isolate of *B. subtilis* and added CS(1g), BHI broth (800 ml), and acetic acid (8 ml) , the flask was placed in an incubator with shaking on 150 rpm at 24 hours , 37 °C in many periods and under sterilized conditions.

Physical Characterization of Prepared Nanoparticles Locally

X-Ray Diffraction (XRD) for Biosynthesized CNPs

XRD was considered foremost an instrument for the analysis and identification of phases in the crystallisation of materials, as well as to determine the properties of constituents in prepared materials. Furthermore, it was used to calculate the mean diameter of nanoparticles. The sample of biosynthesized CNPs was sent to the University of Tehran to conduct of assay.

Fourier Transformed Infrared Spectroscopic (FTIR) for Biosynthesized CNPs

FTIR for biosynthesized CNPs was used to detect the optimum compatibility among drugs and other formulations as nanoparticles, due to its sensitivity to the chemical structure of the nanoparticle's surface, as well as its assistance in detecting active groups besides the bonds in nanoparticles and other materials. FTIR analysis was performed at Tehran University.

Atomic Force Microscopic (AFM) Analysis of Biosynthesized CNPs

The photos of nanoparticles were obtained via atomic force microscopic analysis, which provides a resolution for surfaces of nanoparticles in three dimensions. This technique was used to measure the force exerted by the sample's surfaces on the AFM tip, with a scale of nanonewtons. In the current study, Tehran University analysed the biosynthesized CNPs.

Anticancer Role of Biosynthesized CNPs: A- Culturing and Maintaining of A549 Cell Line

The A549 lung cell line was maintained in Modified Eagle Medium (MEM), supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were passaged using trypsin-EDTA reseeded at 50% to 80% confluence twice a week,

and incubated at 37°C. The A549 Cell line was obtained from the laboratory of cell technologies in Al Harthia, Baghdad, Iraq. B-Cytotoxicity Assay for Biosynthesized CNPs on A549 Cell Line of Lung Cancer: The cell viability assay utilised the compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and was conducted in 96-well plates to determine the cytotoxic effect of the tested compound. Cell lines were seeded at 1×10^4 cells/well. After 24 hours, a confluent monolayer was achieved. Cells were treated with the tested appropriate concentrations of biosynthesized CNPs (1000,500,250,125,62.5,31.25) µg/ml, the cell viability was measured after 72 hours of treatment then the medium was removed and added of 28 µL of 2 mg/mL solution of MTT and placed of the cells in an incubator for 1.5 hour at 37 °C, after removing the MTT solution. The remaining crystals in the wells were solubilised by the addition of 130 µL of Dimethyl Sulfoxide (DMSO), followed by 37°C incubation for 15 minutes with shaking. The absorbance was measured on a microplate reader at a wavelength of 492 nm, and the assay was performed in triplicate. The inhibition rate of cell growth, equivalent to the percentage of cytotoxicity, was calculated using the following equation.

$$\text{Percent (\%)} \text{ of Cell viability} = \left(\frac{\text{Absorbance of treated cell}}{\text{Absorbance of non-treated cell}} \right) \times 100$$

$$\text{Percent (\%)} \text{ of Cytotoxicity} = 100 - \text{Cell Viability.}$$

Statistic Analyze

The current study used Graph Pad Prism (GPP) program version 7 to analyze data obtained where the values were estimated through calculated of $M \pm SE$ in a one-way ANOVA table ($P \leq 0.05$) to know the variance among values in addition to Inferential statistics were achieved by Tukey's multiple comparison test (Mohammed *et al.*,2019; Al-Ziaydi *et al.*,2020).

Results and Discussion

Microscopic Examination and VITEK Detection for Effective Isolate (*S₁*)

An effective isolate was used for the biological synthesis of chitosan nanoparticles, which were diagnosed as *B. subtilis* via observed morphological features and also microscopically examination that it was given characteristics as gram-positive when it was staining with gram stain and it appeared as bacilli form with shaped of rods under microscopically as well as the characteristics of bacterial culture media on nutrient media that seemed to be prominent features of colonies for *B. subtilis* as rough edges with white fuzzy and opaque in fig(1). The effective isolate of *B. subtilis* was diagnosed via biochemical tests with the VITEK 2 Compact System apparatus that provided at least sixty-four tests for the identification of *B. subtilis* at a 93% probability as in table (1).



Figure 1: The Characteristics of Morphology and Microscopy of *B. Subtilis* (A: Gram-stained. B: Nutrient agar)

Table 1: Biochemical Tests of Efficient Isolate (S_1) by VITEK Apparatus.

Patient Name: H						Patient ID: 4602121								
Isolate: 4602121-1(Approved)														
Isolate Number: S ₂														
Card Type: BCL BAR Code: 3209884933041527						Testing Inst: 0001B1B46B5(1858)								
Setup Technologist: Labrotory Administrator (Labadmin)														
Bionumber: 7756222131447														
Quality Organism:						Selected Organism: Bacillus subtilis								
BIOCHEMICAL DETAILS														
1	BXYL	+	3	LysA	+	5	LeuA	+	7	PheA	+	8	ProA	-
9	BGAL	+	10	PyrA	+	12	AlaA	+	13	TyrA	+	14	BMAG	-
15	APPA	(+)	18	CDEX	-	21	GLYG	+	22	INO	+	24	MdG	(-)
25	ELLM	+	26	MdX	-	29	MTE	+	30	GlyA	+	31	dMAN	-
32	dMNE	+	34	Dmlz	-	37	PLE	(-)	39	IRHA	+	41	BGLU	+
43	BMAN	(-)	44	PHC	+	46	AGLU	(-)	47	dTAG	-	48	dTRE	+
50	INU	+	53	dGLU	+	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	+
60	OLD	+	61	ESC	+	63	POLYBA-R	+						

Large Amount Prepared of CNPs by using of B. Subtilis

B. subtilis, had used for the biogenic synthesis of CNPs where it selected an efficient isolate(S_1) illustrating the competence for biosynthesis that occurred extracellular after using a suspension of supernatant and bacterial cells then it was added of CS to the manufacturing of nanomaterial in our study at suitable conditions previously and that can be seen indicators such as changing of the culture medium in appearance shape as in colour to darker as in fig (2) lead to produce of nanoparticles and the activation of bacterial

isolate for biosynthesis, the process of CS synthesis include of CS that be nontoxic and considered agent act against microbial cells according to [12].

On the other hand, the nanoparticles of CS has a role in many fields as from these pharmaceuticals and drugs delivery and others according to the same researcher Van Bavel and his colleagues, also there were methods used to prepare CNPs that rely on essential features of her it's such as degree for deacetylation of CS, weight, charge, size, and crystal shape of CS [13].

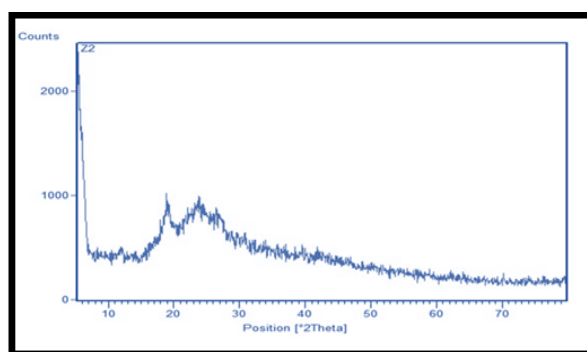
**FIGURE 2:** Biosynthesis of CNPs by Effective Isolate of B. Subtilis on BHIB Medium.

Physical Characterization of Prepared Nanoparticles Locally

X-Ray Diffraction (XRD) for Biosynthesized CNPs

The natural characteristics as the degree of crystallinity for biosynthesized CNPs can be detected by x-ray diffraction which considered the first tool for analysis of the phases of crystalline substances as well as knowing the characteristics of structures, a technique was used to detect the means for nanoparticles diameter and

size of crystalline phase after it was depended on peaks of diffraction, the results found the peak of biosynthesized CNPs at $2\theta = 19.5^\circ$ which was agreed with another study by Jha and Mayanovic, who referred to the occurrence of the peak at $2\theta = 19.6^\circ$, the spectrum of CNPs by X-ray diffraction among 19.5° and 24° where the higher or sharp peak (Z2) in fig (3) referred to CNPs contains other bioorganic materials as proteins [13].

**FIGURE 3:** X-ray Diffraction (XRD) of Biosynthesized CNPs by B. Subtilis.

Fourier Transform Infrared Spectroscopy (FTIR) of Biosynthesized CNPs

The analysis by FT-IR technique is used to detect functional groups present on the surface of CNPs, allowing for the identification of chemical special groups found in the materials and nanoparticles.

The results were in agreement with Dogan and Padmini *et al.* [14, 15]. The spectrum for the band of absorption at wavenumbers 3501.26

cm⁻¹ was related with OH and NH groups, and at 2923.48 cm⁻¹ that associated with (C–H) group, at 1576.20 cm⁻¹ specific to amide (N–H) group, at 1435.72 cm⁻¹ related to the secondary amines (N–H) group, at 1101.57 cm⁻¹ referred to (C–O) group, at 1046.93 cm⁻¹ and 1014.90 cm⁻¹ both related to the (C–N) group that participated with amine groups, and the band of absorption at the wave number 924.71 cm⁻¹, 768.42 cm⁻¹, 649.19 cm⁻¹, and 462.89 cm⁻¹ all these were related with sulfur (S–H) groups fig (4)

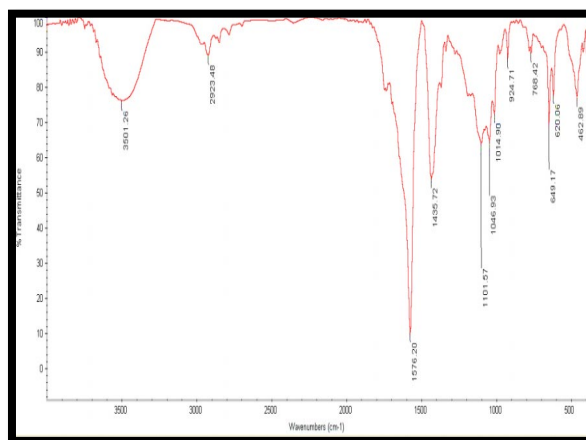


FIGURE 4: FTIR Analysis of Biosynthesized Chitosan NPs locally.

Atomic Force Microscopic (AFM) of Biosynthesized CNPs

The results obtained by AFM analysis provide data about the morphological characteristics of nanoparticles biosynthesised via *B. subtilis*, with a diameter of 47.15 nm (Fig. 5). The images of synthesized nanoparticles can be examined by atomic force microscopic that shows a picture of the material's surface with three-

dimensional and the study of morphology, it can be used to detect some materials that can be combined with biosynthesized CNPs with knowledge of the thickness.

As well as visualized an image with three dimensional, the pictures of molecular structures and scale ways by atomic force that performed by AFM gave a resolution for its [16].

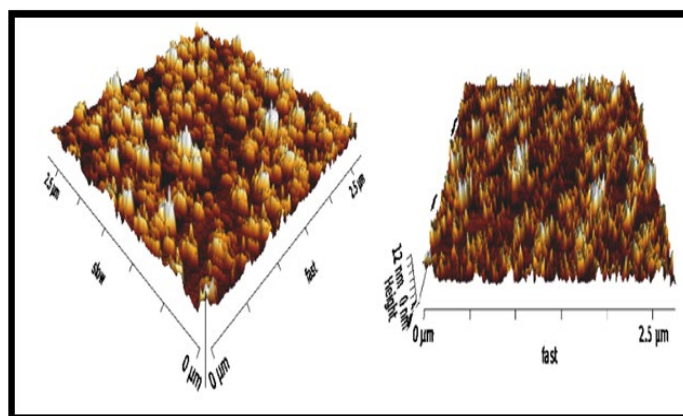


FIGURE 5: Atomic Force Microscopic (AFM) for CNPs Synthesized by *B. Subtilis*.

Anticancer Role of Biosynthesized CNPs

The utilization of 3-(4,5-dimethylthiazal-z-yl) -2,5-diphenyl tetrazolium (MTT) as a colorimetric indicator for changing its color from yellow to purple because of the formation of formazan crystals during the metabolic activity that contains oxidoreductase enzymes which it depended on NADPH as a cofactor where it acted to reduce of MTT led to formation formazan crystals that be was insoluble then it was read her absorbance at 490 nm wavelength, the darker color referred to the number of viable cells that be active metabolically can be showed to know the viability of the cells. The study was used different concentrations of CNPs at 1000, 500, 250, 125, 62.5, and 31.25 μg/ml to know the cytotoxic effect of it on A549 cell line, where

was the cytotoxicity of the cancer cells after 72 hours 58.7 %, 34.3%, 30.3%, 22.3%, 15.5%, and 9.9% respectively as in fig (6). The concentration of IC₅₀ of biosynthesized nanoparticles of chitosan can be detected in the current study in comparison with its different concentrations where a concentration was 2011 μg/ml that referred to suppressing or inhibiting of the cells were treated with synthesized nanoparticles for the A549 cell line range from 435 μg/ml to 9300 μg/ml of substance concentration, the reason for that causing belong to the current study showed as the concentration at 1000 μg/ml showed inhibition effect or cytotoxicity for A549 cell line with percent 58.7 where it was represented high percent compared with another percent of concentrations that used in the current study as in the fig (6) it was

referred previously, fig (7) demonstrated the effect IC₅₀ concentration of synthesized nanoparticles by *B. subtilis* on the A549 cell line of lung

cancer with concentrations at a range 435 to 9300 µg/ml after 72 hours.

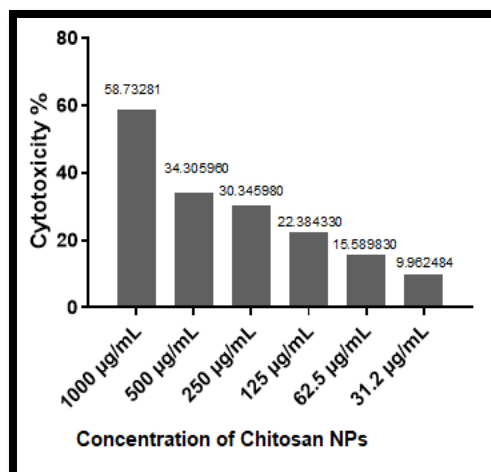


FIGURE 6: Effect of Cytotoxicity on the A 549 Cell Line by Different Concentrations of Biosynthesized CNPs by *B. Subtilis*.

The concentration of IC₅₀ of biosynthesized nanoparticles of chitosan can be detected in the current study in comparison with its different concentrations where a concentration was 2011 µg/ml that referred to suppressing or inhibiting of the cells were treated with synthesized nanoparticles for the A549 cell line range from 435 µg/ml to 9300 µg/ml of substance concentration, the reason for that causing belong to the current study showed as the concentration at 1000 µg/ml

showed inhibition effect or cytotoxicity for A549 cell line with percent 58.7 where it was represented high percent compared with another percent of concentrations that used in the current study as in the fig (6) it was referred previously, fig (7) demonstrated the effect IC₅₀ concentration of synthesized nanoparticles by *B. subtilis* on the A549 cell line of lung cancer with concentrations at a range 435 to 9300 µg/ml after 72 hours.

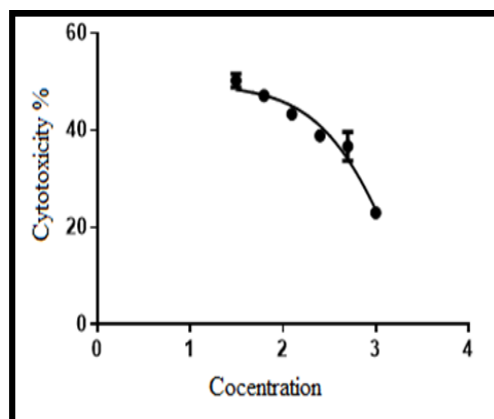


FIGURE 7: Effect of Cytotoxicity of IC₅₀ Concentration from Biosynthesized CNPs by *B. Subtilis* on the A549 Cell Line.

The data from statistical analysis obtained in the current study, when the cell line A549 was treated with a concentration of CNPs at 1000 µg/ml, indicated the presence of a significant difference ($p < 0.0001$) at the 1000 µg/ml concentration. For the other concentrations and the control group, the Tukey's multiple comparisons test was used. There was no significant difference between 500 µg/ml and 250 µg/ml, but a significant difference was found between 500 µg/ml and other concentrations, as well as between 500 µg/ml and the control group. The current study revealed a significant difference between cells treated with a concentration of 250 µg/ml compared with 125 µg/ml ($p = 0.0401$), 62.5 µg/ml ($p = 0.0002$), 31.25 µg/ml, and the control group ($p < 0.0001$), respectively. The current study revealed no significant difference between 125 µg/ml and 62.5 µg/ml ($p = 0.0997$). In comparison, a significant difference was observed between 125 µg/ml and 31.25 µg/ml ($p = 0.0011$) and between 125 µg/ml and the control group ($p < 0.0001$). The cells were treated with a 62.5 µg/ml

concentration, and it appeared that there was no significant difference compared with the 31.25 µg/ml concentration ($p = 0.2304$). However, a significant difference was observed between the 62.5 µg/ml concentration and the control group ($p = 0.0001$). The current study showed was considerable difference between the concentration of 31.25 µg/ml and a control group, fig (8) demonstrated a comparison among different concentrations of these synthesized nanoparticles of chitosan on the A549 cell line.

The micro size of biosynthesized CNPs with their polymeric features was his role in overcoming low bioavailability via making better for each of permeability and solubility of nanoparticles of chitosan as well as other drugs that loaded on it where the chitosan nanoparticles that prepared could cross through narrow junctions among cells, in addition, to induce of properties for permeation and hydrophilic characteristics that assist for solubility and the utilization of anticancer therapies might lead to effects that damage normal cells,

so the utilization of lonely nanoparticles of chitosan or loaded on anticancer therapies act to select of the target organ and it improves the efficiency of therapies for anticancer [17]. The current study suggested that biosynthesized NPs may cause apoptosis for the cell line A549 because these nanoparticles lead to various changes in the morphology as well as inhibition of proliferation cell lines after 72 hours [18]. The nanoparticles act to improve and retain absorption

cellular due to their characteristics of it's as the smallest size that it permits to cross the facilitate entrance into cells via endocytosis as well as playing a role in combining with the functional cellular proteins leading to alteration in the chemical and cellular structure for cell and it also form of reactive oxygen species (ROS) inside cells that causing stress via nucleic acid damage of the cell line leading to death of its [19-21].

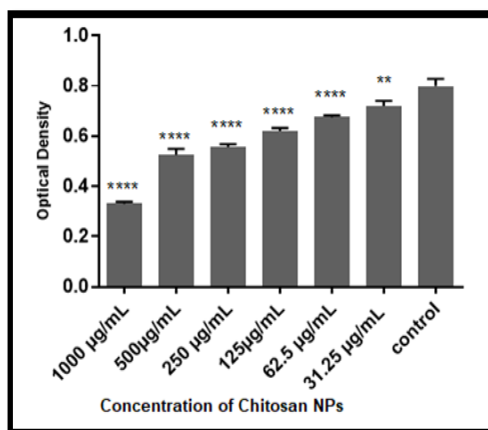


FIGURE 8: A comparison Among Different Concentrations of Biosynthesized Nanoparticles of Chitosan on the A549 Cell Line.

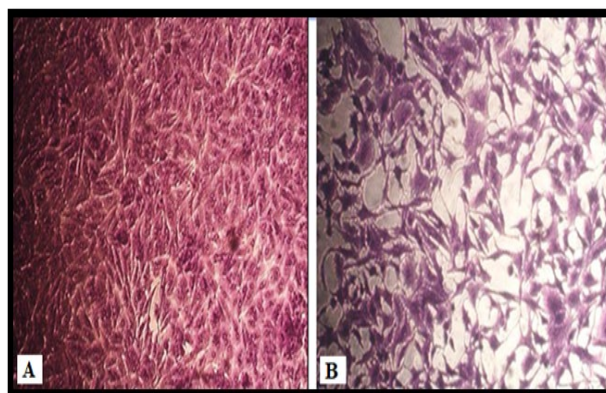


FIGURE 9: Morphological Effect by IC50 of Biosynthesized CNPs by B. Subtilis on the Cell Line A549.

Conclusion

The role of bacterial bacilli as B.subtilis to synthesize CNPs characterized by techniques as XRD to know the degree of crystallinity,

AFM to provide data about morphological characteristics, and FTIR to analyze the surface by noticing the peaks for each specific group for biosynthesized CNPs as well as the nanoparticles synthesized locally can act as agents' activity against cancer cells such as A549 cell line.

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